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BIODEGRADATION OF OIL IN SEAWATER FOR NAVAL POLLUTION CONTROL

by

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13. ABSTRACT

The report describes the isolation and utilization of pure and mixed microbial cultures for experiments on the biodegradation of crude oil Bunker C fuel and marine diesel. Many microbial species were found that had hydrocarbonoclastic activity. When pure cultures were combined in mixtures the activity was much greater, 91% oxidation in seven days, than the activity of any one of the component species when used in a pure culture.

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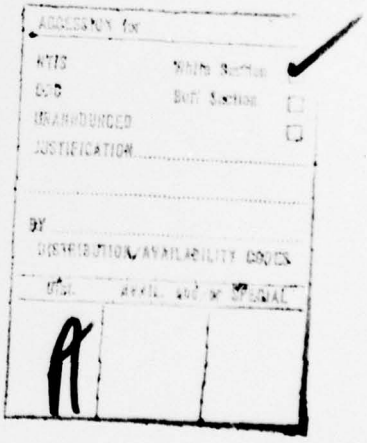

INTRODUCTION

The problem of oil spillage is one of great magnitude and concern to the Navy, particularly in this era of increasing legal restrictions and ecological awareness. Oil on the seas is a fire hazard and a menace to organisms of the marine environment as well as an offense against aesthetics to those who use the seas for recreation and inspiration.

The negative consequences of oil spillage on waters and beaches are not new. They are concomitant with the origin and development of propulsion units and other devices utilizing fossil fuels. Pertinent to the context of this report, it is imperative to maintain that natural oil seeps from the ocean floor have undoubtedly existed for millennia. Merz (1959) reported on the almost universal presence of oil, derived from natural oil seeps, on the beaches of Southern California. The amounts of oil cited are small, approximately 60cc per $50m^2$, and generally non-persistent. Coal Oil Point, in the Santa Barbara Channel, is an extreme example of a beach having continued deposits of large amounts of crude oil and tar. Allen, et al (1970) determined this spillage to be on the order of 50 to 70 barrels (8,000 to 11,000 liters) of oil per day into the channel and on the beaches. The beach deposits are estimated to be greater than any other beach in Southern California.

As well known as the appearance of oil and tar on the beaches and adjacent waters is its disappearance after unspecified periods of time. ZoBell (1969) reported that oil-oxidizing bacteria are often abundant in marine sediments and seawater of coastal areas where natural or man-induced pollution is persistent. ZoBell further noted that oil-oxidizing bacteria, yeasts, and molds have been identified in water and mud samples in many parts of the world.

The present report is concerned with the measurable activity of micro-organisms to oxidize certain fossil fuels. The organisms utilized were isolated from beaches and inshore marine waters where oil spills are common or were secured from type culture collections.

PROCEDURE

The literature, from 1943 to the contemporary period, dealing with microbial oxidation of hydrocarbons was searched to prepare a listing of micro-organisms reported to possess an ability to degrade hydrocarbons. Subsequently, where possible, lyophilized cultures were secured from type culture collections, notably the American Type Culture Collection of Rockville, Maryland.

Other sources of micro-organisms were the local beaches and harbor areas where oil spills are common; e.g., Coal Oil Point, Carpenteria Beach, and Los Angeles-Wilmington Harbor. Aliquot samples of beach sand or harbor soil or water were placed in a mineral salts medium made with aged filtered sea water and containing 0.75 percent hydrocarbon, either crude oil, marine diesel or Bunker C fuel as a sole source of organic material. Incubation was at room temperature, 20°C-25°C, upon a rotary shaker and varied from periods of one week to ten days. During the periods of incubation microbial populations were determined daily, by plate counts, and hydrocarbon oxidizing micro-organisms were isolated.

The isolated pure cultures were maintained on optimal media and transferred as required. Prior to the utilization of the cultures they were transferred to broth and during the late exponential growth phase the cells were centrifuged and washed three times with the mineral salts medium to remove the major portion of the organic matter of the media. In the experiments approximately 3×10^{12} cells were used as an inoculum in 250ml flasks containing 150ml of mineral salts medium containing 0.75% of hydrocarbon as the sole source of organic matter. Controls were identical flasks without added micro-organisms. The flasks were incubated at room temperature upon a rotary shaker for seven days. At the end of the incubation period, the quantity of hydrocarbon remaining was determined by an extraction process.

In the extraction procedure, several washings of chloroform were used as the hydrocarbon solvent. The extract was dried over anhydrous sodium sulfate, filtered to remove the drying agent and evaporated to dryness under reduced pressure. Evaporation was continued until three consecutive weighings were identical to the nearest milligram.

Initial experiments utilized pure cultures of one microbial species and subsequent experiments involved mixtures of two or more pure cultures in single flasks.

Over the period of experimentation many media modifications were tested as were other conditions that might optimize hydrocarbon oxidizing activity.

A longevity study was also performed. In these experiments pure cultures of one or more microbial species were added to a mineral salts hydrocarbon mixture and incubated for a period of a year with occasional additions of mineral salts solution to maintain an appropriate level of liquid.

RESULTS

In the period of experimentation, the media and conditions of

experiments significantly changed. This represents an increasing knowledge of parameters that facilitate microbial oxidation of hydrocarbons. For example, the use of ammonium salts as a nitrogen source lowered the pH of sea water used in the media from a pH of 8 to a detrimental pH of 4 as the bacteria multiplied. The unfavorable pH was a principal factor in the reduction of microbial population. The use of buffer solutions alleviated this condition. The addition of sterile air to flasks increased microbial activity approximately seven-fold. The incorporation of sterile diatomaceous earth to flasks increased adsorptive surface air for hydrocarbon and microbes and increased activity three-fold.

Fifty-five species of hydrocarbon oxidizing species of micro-organisms were secured from type culture collections and 62 species were isolated from local habitats. The maintenance of this comparatively large collection of micro-organisms is time consuming for they must be transferred monthly to fresh media.

Many of the organisms previously cited in the literature as having hydrocarbon-oxidizing ability and secured from culture collections failed to demonstrate activity under the conditions of the present experiments. That all media were prepared using salt water (75%) and fresh water (25%) rather than fresh water alone might well explain lack of microbial activity. Possibly the microbes could not oxidize the specific hydrocarbons used in these experiments. The species not demonstrating any hydrocarbonoclastic activity are:

Achromobacter delicatulus

Arthrobacter ureafaciens

Brevibacteruim pusillum

Candida parapsilosis

Flavobacterium arborescens

Hydrogenomonas facilis

Hyphomicrobium indicum

Micrococus varians

Mycobacterium rubrum

Mycobacterium smegmatis

Pseudomomas cruciviae

Pseudomonas dacunhae

Pseudomonas stutzeri

Serratia marinorubra

Vibro cyclosites

The pure cultures of microorganisms that demonstrated a significant hydrocarbonoclastic actively are:

Organism	Hydrocarbon Oxidized (% in a 7-day period)		
	Crude Oil	Bunker C	Marine Diesel
Achromobacter cycloclastes	23	12	8
Aeromonas hydrophlta subsp formicans	8	11	4
Arthrobacter arthrobotryoides	44	22	12
Arthrobacter citreus	9	3	0
Arthrobacter simplex	31	27	19
Azotobacter chroococcum	28	12	8
Azotomonas insoluta	13	9	3
Bacillus insolitus	20	9	7
Candida lipolytica	41	33	17
Candida petrophilum	37	22	19
Cellulomonas fimi	12	7	3
Cephalosporium acremonium	18	11	8
Clasdosporium resinae	18	4	9
Corynebacterium hydrocarboclastus	32	27	18
Cunninghamella elegans	67	32	29
Flavobacterium ferrungineum	17	9	5
Flavobacterium marinotypicum	51	26	16
Hyphomicrobium neptunium	39	15	11
Micrococcus roseus	23	7	12
Micrococcus parrafinolyticus	32	11	9
Mycobacterium hyalinum	17	4	4
Mycobacterium phlei	17	9	8
Mycobacterium rhodochrous	26	11	12
Mycoplana bullata	30	19	5

Mycoplana dimorpha	26	13	8
Nitrobacter agilis	12	7	4
Nocardia corralina	31	22	8
Nocardia hydrocarbonoxydans	36	19	17
Nocardia opaca	29	11	10
Nocardia parrafinica	32	10	8
Nocardia petreoleophila	30	17	6
Penicillium zonatum	42	26	8
Pseudomonas boreopolis	24	12	7
Pseudomonas creosotensis	27	10	6
Pseudomonas desmolytica	29	11	9
Pseudomonas facilis	22	8	7
Pseudomonas oleovorans	18	14	11
Pseudomonas resinovorans	20	12	7
Pseudomonas rubescens	12	4	6
Pseudomonas putida	19	11	8
Sarcina aurantica	6	0	0
Sarcina flava	12	7	4
Vibrio sp	16	4	5
Vibrio neocistis	18	9	3

The selection of microbial species for experiments involving more than one species was based on performance in the above described exercises utilizing but one species. The components of the mixed cultures were individually grown as pure cultures in broth, centrifuged and washed, and then added to the flasks containing mineral salts medium and hydrocarbon. The combined total of all microorganisms added was approximately 3×10^{12} cells.

In some experiments the total of hydrocarbons oxidized did not exceed that of either component when used alone. In such a category are the mixed cultures of Arthrobacter simplex and Bacillus insolitus, Corynebacterium hydrocarboclastus and Candida petrophilum, and Micrococcus roseus with Nocardia corralina.

More encouraging are those combinations of cultures whose activity is greater than either of the component cultures individually, or greater than both collectively. Some examples of these results are as follows:

Organism	Hydrocarbon Oxidized (% in a 7-day period)		
	Crude Oil	Bunker C	Marine Diesel
Corynebacterium hydrocarboclastus and Candida petrophilum	63	31	24
Flavobacterium marinotypicum and Hyphomicrobium neptunium	57	29	25
Arthrobacter arthrobotryoides and Candida lipolytica	61	34	31
Arthrobacter simplex and Arthrobacter arthrobotryoides	44	22	17
Candida petrophilum and Flavobacterium marinotypicum	54	29	21
Hyphomicrobium neptunium and Arthrobacter simplex	51	18	11
Candida petrophilum and Arthrobacter arthrobotryoides	64	39	18
Micrococcus paraffinolyticus and Arthrobacter arthrobotryoides	67	41	27
Mycobacterium rhodochrous and Arthrobacter arthrobotryoides	85	47	32
Mycoplana bullata and Pseudomonas desmolytica	43	27	34

Cunninghamella elegans and *Arthrobacter simplex*	79	53	21
Cunninghamella elegans and *Arthrobacter arthrobotryoides*	87	42	38
Nocardia paraffinica and *Penicillium zonatum*	54	17	28
Vibrio sp and *Cunninghamella elegans*	62	40	19
Nocardia hydrocarbonoxydans and *Arthrobacter arthrobotryoides*	58	15	21
Candida lipolytica and *Cunninghamella elegans*	61	52	18
Corynebacterium hydrocarboclastus and *Pseudomonas desmolytica*	82	39	17

Several experiments utilized combinations of three or four cultures. The procedure was similar to that employing two cultures. With a single exception, the mixtures yielded results no better than mixtures involving but two species. The single exception was a mixture of *Cunninghamella elegans*, *Arthrobacter arthrobotryoides*, *Nocardia hydrocarbonoxydans*, and *Pseudomonas desmolytica*, which in seven days oxidized 91%, 73%, and 50% of crude oil, Bunker C, and marine diesel, respectively.

In those experiments allowed to incubate for a year at room temperature, the bacteria reached a peak activity after from 10 to 14 days and subsequently the activity significantly dropped, but nevertheless did continue at a slow rate for the entire year.

CONCLUSIONS

There appears to be no dearth of microbes, including bacteria, fungi, and yeasts that have the capacity to oxidize crude oil, Bunker C fuel, and marine diesel. In most experiments the percentage of crude oil oxidized

exceeded the percentage of Bunker C oxidized in the same period. In general, Bunker C was oxidized at a faster rate than marine diesel.

Many organisms that demonstrated an ability to survive in media with a hydrocarbon as a sole source of organic matter lacked a perceptible ability to degrade measurable amounts of hydrocarbon. Commonly, the combination of different species was more active than the activity of either of the component species alone. This effect appears to be synergistic. On the contrary, an antagonistic effect also occurs when the sum total of hydrocarbon degeneration is less than that achieved by any component of a mixed culture when used alone as a pure culture.

The author concludes that the continued endeavor of putting together different combinations of cultures will ultimately yield a mixture that, with appropriate nutrients, can be added in a lyophilized form to an oil spill, after physical methods of clean-up have been completed, to eliminate the last vestiges of oil.

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